Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3888–3892, June 1984
Neurobiology

Antibodies against γ-aminobutyric acid: Specificity studies and immunocytochemical results

(glutaraldehyde/reducing agent/RIA system/hippocampus/cerebellum)

PHILIPPE SEGUELA*, MICHEL GEFFARD*, RUUD M. BUIJS†, AND MICHEL LE MOAL*

*Institut de Biochimie Cellulaire et Neurochimie du Centre National de la Recherche Scientifique, 1 rue Camille Saint-Saëns, 33077 Bordeaux cedex, and Institut National de la Santé et de la Recherche Médicale, Unité 259, Domaine de Carreire, 33076 Bordeaux cedex, France; and †Netherlands Institute for Brain Research, Ijdijk 28, 1095 DB Amsterdam, The Netherlands

Communicated by Floyd E. Bloom, February 14, 1984

ABSTRACT **Antibodies against γ-aminobutyric acid (GABA)-glutaraldehyde-lysine were obtained by using a procedure based upon (*i*) a high yield of coupling of GABA to protein carriers, (*ii*) the reduction of the resulting immunoreactive double bonds, and (*iii*) a protocol of alternative immunizations using different immunogens having in common only the GABA-glutaraldehyde-lysine segment. This strategy led to the use of the resulting GABA antiserum without further purification. Specificity controls have been carried out with a radiolabeled ligand, [^{3}H]GABA-glutaraldehyde-prolylphenylalanyl-lysine, which mimicked the structure of the immunogen and the fixed hapten in the tissue. Displacement curves showed that the nearest coupled analogs, β-alanine and glycine, cross-react poorly with GABA, requiring 175-fold or 795-fold higher concentrations, respectively. Immunocytochemical results indicated that the localization obtained with this GABA antiserum largely corresponds with that reported after glutamate decarboxylase immunocytochemistry. The approach may have general applicability to other small molecules such as amino acids.**

Neurobiology needs specific tools to attack the problem of neurotransmitter localization in the central nervous system. Apart from indolamines (1), cyclic AMP and GMP (2), it has been difficult to obtain antibodies directed against small haptens such as amino acids and catecholamines. Recently, however, sensitive and specific anti-dopamine antibodies were raised in our laboratory by respecting the following methodological principles (3). First, the coupling mechanism used during the synthesis of the immunogen must preserve the distinguishing part of the molecule and the same procedure should preferably be used to fix the molecule to the tissue. Second, the immune response must be directed selectively against the coupled hapten. Third, the final antigenic structure must be identical to the antigens fixed to the tissue for antibodies to be used in immunocytochemical studies.

By using these procedures, a perfect correlation between the radioimmunological studies and the immunocytochemical applications was found. For the localization of γ-aminobutyric acid (GABA), one of the main inhibitory transmitters in the central nervous system (4, 5), one has until now had to rely on the localization of the synthesizing enzyme glutamate decarboxylase (6–8), which can then be used as an indirect marker for GABA. Apart from the fact that this is an indirect procedure to demonstrate GABA, the isolation and purification of this enzyme represents an enormous task.

Therefore, one of the objectives of this study was to raise specific and sensitive antibodies against GABA; as a result, a procedure is described that led to obtaining a highly specific GABA antiserum suitable for immunocytochemistry.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Synthesis of Immunogenic Conjugates. Ten milligrams of GABA and 2 μl of [^{3}H]GABA in 1 ml of distilled water were mixed with 1 ml of 3 M acetate buffer (pH 7.8) containing either 30 mg of bovine serum albumin (hereafter termed albumin) (Sigma), 30 mg of bovine Hb (Sigma), or 30 mg of poly(L-lysine) (Peninsula Laboratories). The reaction was started by adding 1 ml of 5% glutaraldehyde and lasted 3 min at room temperature. Glutaraldehyde was chosen for its high yield of coupling with the ε-amino group of lysyl residues in proteins (9, 10). The orange-yellow color and a stable pH around 7 indicated that the coupling reaction was complete; 1 ml of a sodium borohydride (Merck) solution (10 mM) was added to saturate the double bonds. After reduction, the mixture turned from orange-yellow to translucent. The solution was then dialyzed at 4°C and the precipitate was removed by centrifugation. β-Radioactivity and the weight of 1 ml of lyophilized conjugate were determined, allowing molar ratios to be calculated [GABA/albumin = 21, GABA/Hb = 50, and GABA/poly(L-lysine) = 34].

Synthesis of Radiolabeled Ligand. As described for dopamine (3), glutaraldehyde was used to link the ε-amino group of proline-phenylalanine-lysine (Bachem Fine Chemicals, Torrance, CA) to the amino group of GABA. Pro-Phe-Lys was chosen for two main reasons: (*i*) only the ε-amino group of lysine was free; and (*ii*) phenylalanine had an ultraviolet spectrum that permitted the determination of the molar concentration of Pro-Phe-Lys after coupling, and thus an index of the molar ratio between GABA, or other amino acids, and Pro-Phe-Lys. The coupling reaction of [^{3}H]GABA (Amersham) to Pro-Phe-Lys was carried out in the same way as the coupling to the immunogenic conjugates. Briefly, 500 μl of 0.1 M Pro-Phe-Lys (pH 8), 100 μl of 3 M acetate buffer, and 40 μl of [^{3}H]GABA solution were mixed. The reaction was started by mixing 100 μl of a 5% glutaraldehyde solution.

The mixture was left for 3 min and then was saturated with sodium borohydride for 30 min. The mixture was diluted with 1 ml of phosphate buffer before loading onto a quaternary ammonium ethyl-Sephadex column (Pharmacia). The column (1 × 48 cm) was eluted with 400 ml of a 10 mM–0.8 M NaCl gradient in 10 mM phosphate buffer (pH 6.2).

Synthesis of Amino Acid Derivatives. GABA, glycine, β-alanine, glutamate, aspartate, and taurine were coupled to Pro-Phe-Lys with glutaraldehyde under the same conditions as the radiolabeled ligand. After quaternary ammonium ethyl-Sephadex chromatography, the concentration of each derivative was calculated by spectral analysis and β-radioactivity counting. The molar ratio was calculated and GABA,

Abbreviation: GABA, γ-aminobutyric acid.

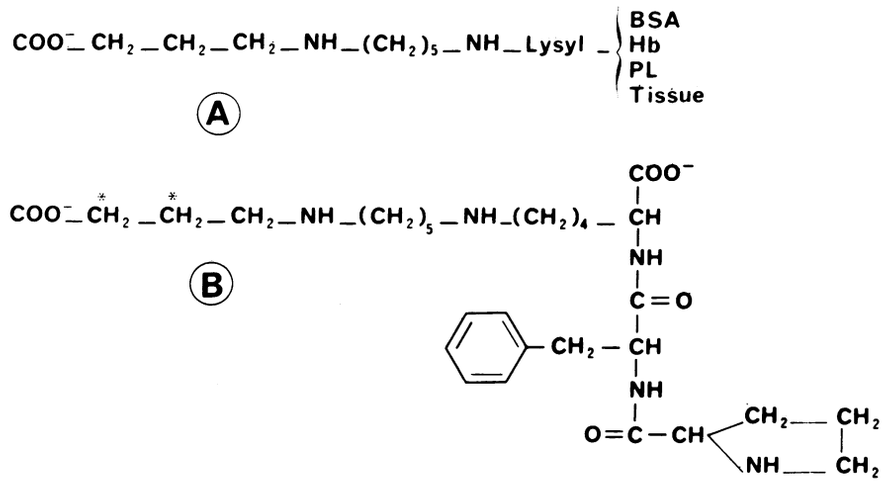

FIG. 1. Structural analogy between hapten coupled to protein carriers [albumin, BSA; Hb; poly(L-lysine), PL; or tissue] (*A*) and radiolabeled ligand [^{3}H]GABA-glutaraldehyde-Pro-Phe-Lys (*B*).

glycine, glutamate, aspartate, and taurine conjugated concentrations were determined.

Immunization. Two rabbits were immunized with each immunogen successively according to the method of Vaitukaitis *et al.* (11) with slight modifications. The first booster injected contained 500 μg of GABA-glutaraldehyde-albumin emulsified in 500 μl of 0.15 M NaCl and 750 μl of complete Freund's adjuvant (Difco). The second booster injection consisted of GABA-glutaraldehyde-Hb, the third of GABA-glutaraldehyde-poly(L-lysine), the fourth of GABA-glutaraldehyde-albumin, and so on. Immunization was carried out for 4 months.

Radioimmunological Incubation. These were performed by equilibrium dialysis (2, 12). The dialysis cells were fitted with large-pore cellulose membranes (Sartorius). They were rinsed with distilled water before re-use. Each concentration of amino acid conjugate was mixed with an equal volume of radiolabeled ligand before being placed on one side of the membrane; the diluted antibody was placed on the other side. The incubation solution, 0.1 M citrate buffer (pH 6.2), contained 1 mg of albumin per ml and 10 mM sodium azide. The final volume was 300 μl (2 × 150 μl), and the incubation time was 20 hr at 4°C.

Imunocytochemical Procedure. Rat brains were perfusion-fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5). The brain was sectioned in slices of about 4 mm and was postfixed in the same fixative for 1.5–2 hr. Vibratome sections (50 μm) were incubated in GABA antiserum that was diluted 1:1500; this antiserum was visualized by using the peroxidase-antiperoxidase (13) method with diaminobenzidine as chromogen. Further details on the immunocytochemical procedure have been described (14).

RESULTS

GABA Antiserum Titer. The structural analogy between the synthesized immunogens and the radioactive ligand (Fig. 1) allowed us to follow the rise of the antibody titer with time in the RIA system. The maximal titer was reached after the ninth immunization, which corresponds to the fourth month (Fig. 2). The serum having the highest titer was used both for obtaining displacement curves and for the immunocytochemical applications.

GABA Antiserum Specificity. Unmodified GABA was unable to displace the radiolabeled ligand, even at a concentration of 10 μM. However, GABA coupled to Pro-Phe-Lys with glutaraldehyde yielded an excellent binding, which

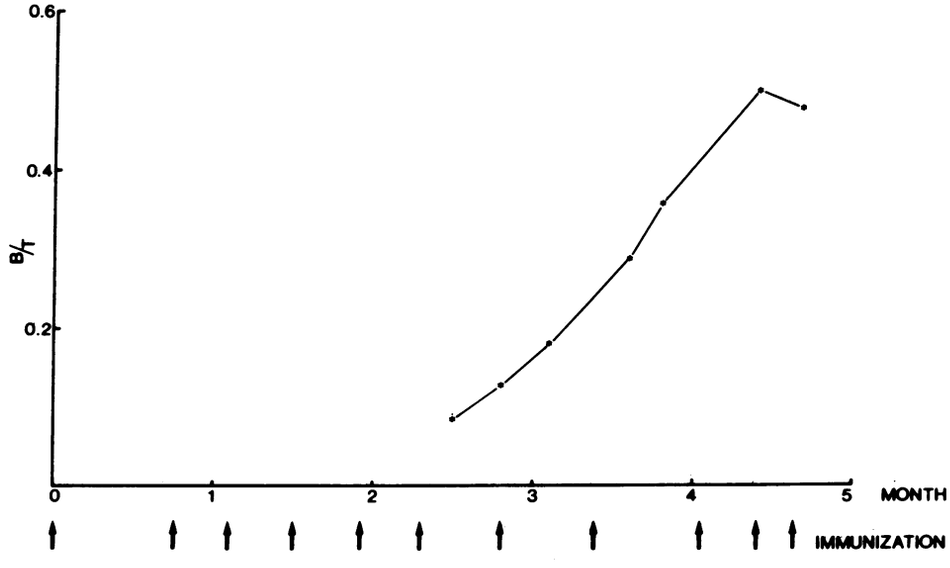

FIG. 2. Time course of immunization and GABA antiserum titers. The rabbit received successively a booster injection and an intramuscular injection. Serum dilution was 1:500. Antiserum binding was tested in equilibrium dialysis, with [^{3}H]GABA-glutaraldehyde-Pro-Phe-Lys fully recognized by antibodies. B/T: bound/total ratio.

demonstrated that the glutaraldehyde chain was important for the immunorecognition. The specificity of the GABA antiserum was quantitatively demonstrated by using also [^{3}H]GABA-glutaraldehyde-Pro-Phe-Lys. Fig. 3 shows the displacement curves of [^{3}H]GABA-glutaraldehyde-Pro-Phe-Lys produced by different amino acids coupled in the same way. The linear and unsubstituted compounds were recognized by the antibody; however, β-alanine and glycine were recognized 175 and 795 times, respectively, less than GABA-glutaraldehyde-Pro-Phe-Lys. These values eliminated all ambiguities in the specific applications of the anti-GABA antibodies. No binding was detected with the coupled and substituted amino acids (aspartate and glutamate) at concentrations between 1 nM and 10 μm. In addition, the recognition for coupled taurine was approximately the same as the one found for coupled aspartate and glutamate (see Table 1). We explain this absence of cross-immunoreactivity by the considerable structural difference that exists between GABA and taurine at their free terminal functions.

Contrary to findings with previously obtained antisera (15), purifications on solid phase were not necessary to eliminate nonspecific immunoglobulin G populations in sera directed against nonreduced immunogens.

Immunocytochemical Results. An extinction in staining was observed after the native serum was adsorbed with an immunogenic solution [GABA-glutaraldehyde-(Hb or albumin)]. A dramatic decrease in staining was noticed after adsorption with a 1 mM free GABA solution, confirming the major role of the GABA terminal part in immunorecognition. Neither qualitative nor quantitative difference in staining appeared after the serum was mixed with the following coupled proteins: glutamate-glutaraldehyde-albumin aspartate-glutaraldehyde-albumin, taurine-glutaraldehyde-albumin, glycine-glutaraldehyde-albumin and β-alanine-glutaraldehyde-albumin.

By using the anti-GABA antibodies on glutaraldehyde-fixed sections, an intense staining was revealed in fibers and cell bodies in many brain regions. It appeared that even without the use of colchicine, many reactive cell bodies were readily visible.

As previously described, the cerebral cortex (Fig. 4*A*), the hippocampus, in which the innervation of the granular and pyramidal cells was most prominent (Fig. 4*B*), and the cerebellar cortex (Fig. 5*A*) appeared to contain GABA. In the cerebellar cortex, Golgi, basket, Purkinje, and stellate cells were immunoreactive (Fig. 5*B*).

Table 1. Cross-reactivity ratio of coupled amino acids and uncoupled GABA

Compound	Ratio*
GABA-glutaraldehyde-Pro-Phe-Lys	1
β-Alanine-glutaraldehyde-Pro-Phe-Lys	1:175
Glycine-glutaraldehyde-Pro-Phe-Lys	1:795
Taurine-glutaraldehyde-Pro-Phe-Lys	1:17,700
GABA	1:17,700
Aspartate-glutaraldehyde-Pro-Phe-Lys	<1:50,000
Glutamate-glutaraldehyde-Pro-Phe-Lys	<1:50,000

*GABA-glutaraldehyde-Pro-Phe-Lys concentration coupled amino acid or GABA concentration at half-displacement.

DISCUSSION

Since the pioneering work of Landsteiner (16), who coupled small haptens to protein carriers (e.g., ε-dinitrophenyllysine), it has been possible to obtain specific antibodies against small molecules. In neurobiology, successful antibodies were recently developed against neurotransmitters having a cyclic structure, such as indolamines (1) and catecholamines (3). Consequently, it became conceivable to develop antibodies against small linear chain molecules such as GABA. The procedure to couple antigens to protein carriers as described in the present paper allowed the induction of a specific and sensitive antibody directed against GABA-glutaraldehyde-lysine. Several reasons might account for this result. First, the high yield of the coupling reaction resulted in a protein carrier loaded with many GABA-glutaraldehyde-lysine determinants. By this coupling procedure, the immune response was directed against antigenic determinants (GABA-glutaraldehyde-lysine), of which large amounts were found in immunogen. Second, an immunization strategy was carried out by using three different protein carriers to confine immunoreactivity towards GABA-glutaraldehyde-ly-

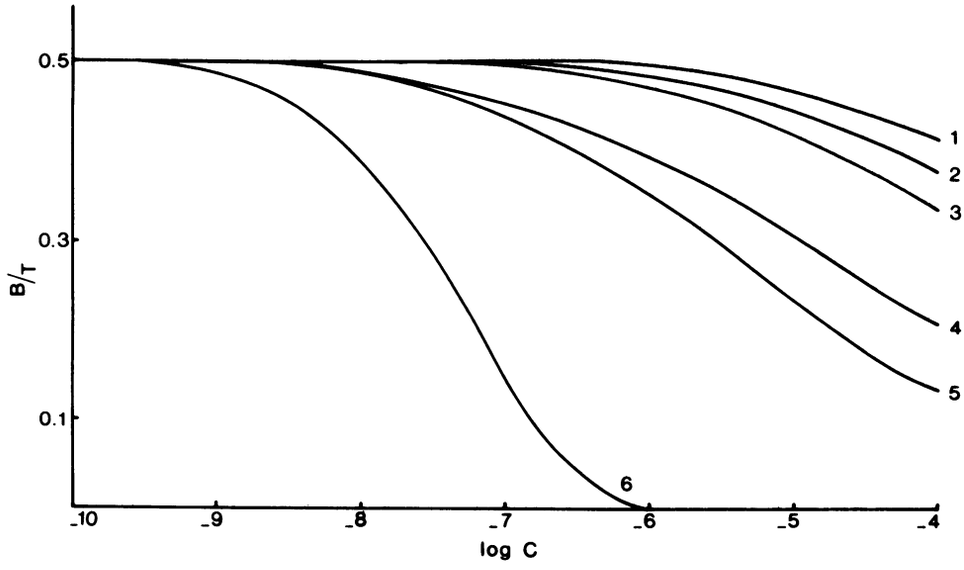

FIG. 3. Displacement curves of [^{3}H]GABA-glutaraldehyde-Pro-Phe-Lys by uncoupled GABA and coupled amino acids. Curve 1, glutamate-glutaraldehyde-Pro-Phe-Lys; curve 2, aspartate-glutaraldehyde-Pro-Phe-Lys; curve 3, uncoupled GABA and taurine-glutaraldehyde-Pro-Phe-Lys; curve 4, glycine-glutaraldehyde-Pro-Phe-Lys; curve 5, β-alanine-glutaraldehyde-Pro-Phe-Lys; curve 6, GABA-glutaraldehyde-Pro-Phe-Lys. This experiment was done at an antibody dilution of 1:500 in 0.1 M citrate buffer (pH 6.2) containing 1 mg of albumin per ml and 10 mM sodium azide. Equilibrium dialysis lasted overnight at 4°C. β-Radioactivity of 100 μl was counted on each side of the membrane to calculate bound/total ratios (B/T).

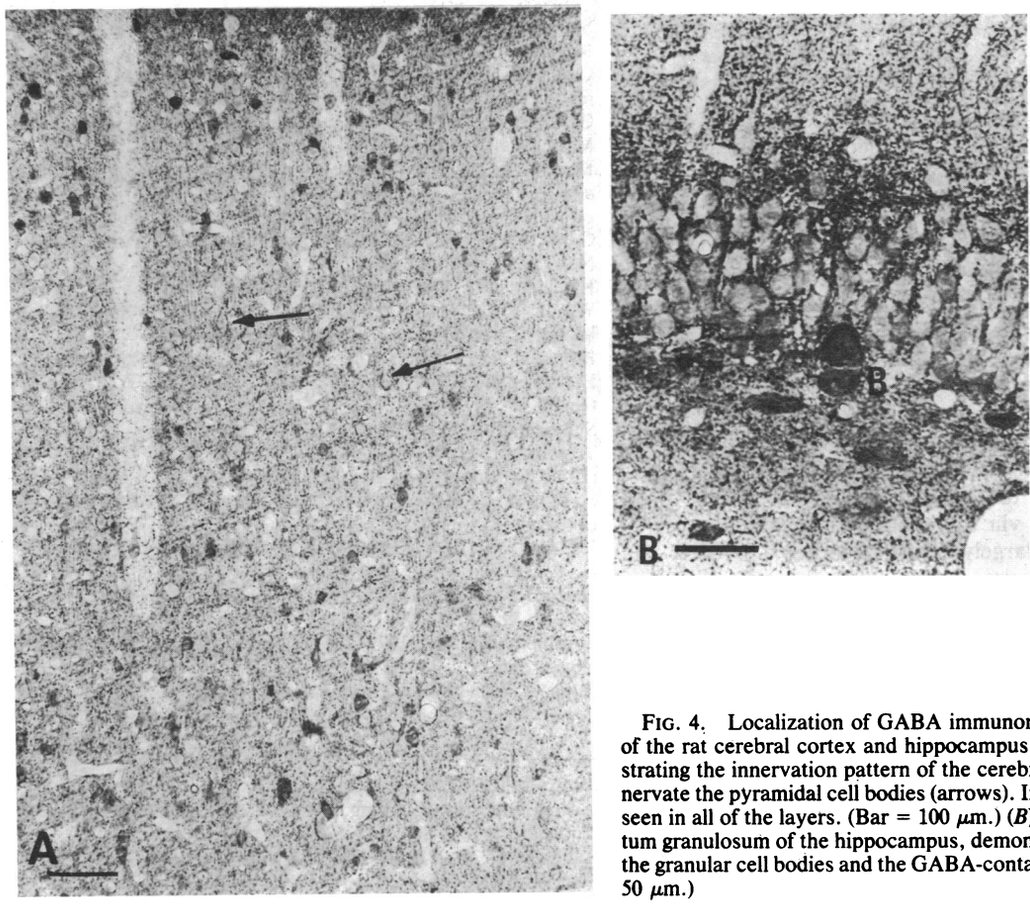

FIG. 4. Localization of GABA immunoreactivity in transversal sections of the rat cerebral cortex and hippocampus. (*A*) Low magnification demonstrating the innervation pattern of the cerebral cortex. GABAergic fibers innervate the pyramidal cell bodies (arrows). In addition, a dense innervation is seen in all of the layers. (Bar = 100 μm.) (*B*) High magnification of the striatum granulosum of the hippocampus, demonstrating the dense innervation of the granular cell bodies and the GABA-containing basket neurons (B). (Bar = 50 μm.)

sine, the only common antigenic determinant. Third, double bonds of the Schiff's base induced by glutaraldehyde coupling were reduced by sodium borohydride; otherwise antigenicity would be directed only against redundant lysine residues. Nonreduced immunogens led to obtaining antisera having nonspecific immunoglobulin Gs directed against imines. Several purifications on solid phase were necessary to eliminate them (15). This procedure indicated the importance of imine or double bond immunoreactivity in the haptenic chain. Reduction seemed to be a decisive step to obtain a high native serum specificity.

Due to all of these precautions, the immune response was probably largely directed against GABA-glutaraldehyde-lysine complexes. It appeared that the affinity and specificity of our anti-GABA antiserum were more important than recently reported (15). This conclusion came from the fact that antibodies recognized [^{3}H]GABA only when coupled with glutaraldehyde. In addition, specificity studies demonstrated that β-alanine had the highest cross-reacting capacity, its cross-reactivity ratio being 1:175. In this respect, it was interesting to note that the cross-reactivity with glutamate was <1:50,000. This result could be explained by the fact that the

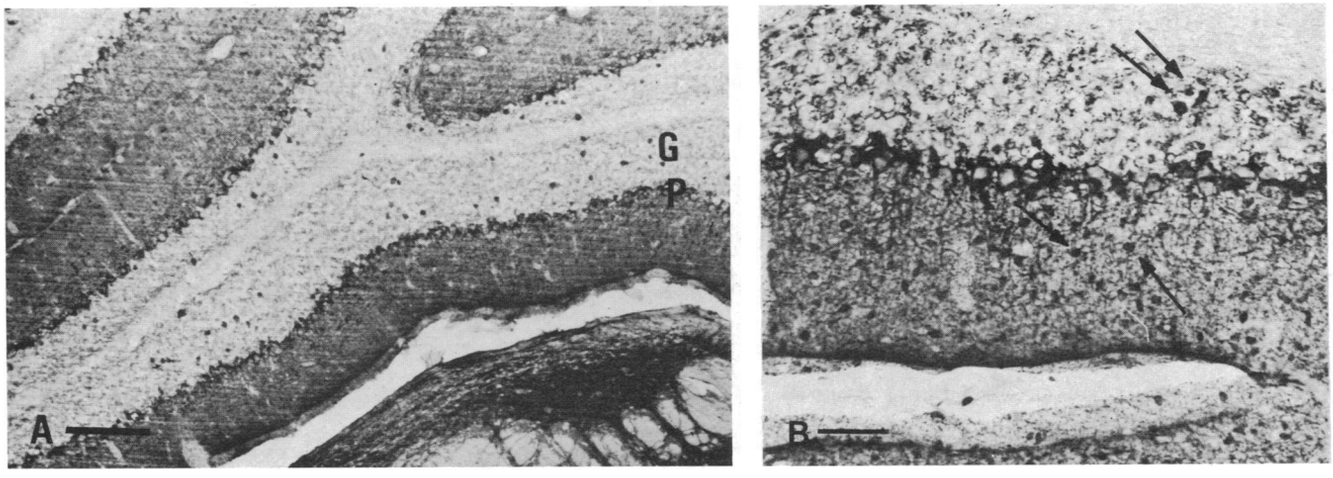

FIG. 5. Localization of GABA immunoreactivity in a transversal section of the rat cerebellum. (*A*) Low magnification, demonstrating the GABA-containing Golgi neurons in the granular layer, the innervation of the Purkinje cell bodies (P), and the dense innervation of the molecular layer. (Bar = 500 μm.) (*B*) Higher magnification, demonstrating GABA-containing Golgi neurons (double arrows) and stellate cell bodies (single arrows). Note the innervation of the Purkinje neurons and the difference in innervation pattern between the molecular and granular layer. (Bar = 100 μm.)

major difference of glutamate with GABA was the addition of a carboxyl group, whereas the difference with β-alanine was only in length. From these specificity studies, it was possible to determine by Scatchard analysis a dissociation constant (K_d) of 2.10×10^{-8} M for GABA-glutaraldehyde-lysine with the antibody. From this K_d, the standard free enthalpy was calculated to be −9.8 kcal/mol (1 cal = 4.184 J). In addition, affinity and specificity of this antibody were correlated with the immunocytochemical results.

The ubiquitous presence of GABA in the central nervous system was confirmed directly and the localization obtained in this study matched perfectly that reported with glutamic acid decarboxylase immunocytochemistry (6–8). The only exception was that with this GABA antibody, cell bodies could be observed without colchicine treatment, whereas in glutamic acid decarboxylase immunocytochemistry, the Purkinje cells were only visible after this treatment (8). The latter result emphasizes again the need to demonstrate the transmitter directly and not via the synthesizing enzyme. However, the present study largely confirms earlier reports (6) that GABA neurons innervate nearly every part of the central nervous system. In addition, this immunocytochemical procedure will, because of its fixation with glutaraldehyde, greatly facilitate future electron microscopical studies on the ultrastructural localization of this amino acid transmitter.

The authors thank Mrs. Anne-Marie Heinrich and Rose-Marie Bluthe for their excellent technical assistance and Mr. Anthony Heape for reading the manuscript. This work was supported by Grant DGRST-MRI 81 E-0573.

1. Steinbusch, H. W. M., Verhofstad, A. A. J. & Joosten, M. W. J. (1978) *Neurosciences* **3,** 811–819.
2. Cailla, H. L., Cros, G. S., Jolu, E. J., Delaage, M. A. & Depieds, R. C. (1973) *Anal. Biochem.* **56,** 383–393.
3. Geffard, M., Buijs, R. M., Seguela, P., Pool, C. W. & Le Moal, M. (1984) *Brain Res.* **294,** 161–165.
4. Kravitz, E. A. (1967) in *Acetylcholine, γ-Aminobutyric Acid and Glutamic Acid: Physiological and Chemical Studies Related to Their Role as Neurotransmitter Agents*, eds. Quarton, G. C., Melnechuk, T. & Schmitt, F. O. (The Neurosciences, New York), pp. 433–444.
5. Krnjevic, D. (1974) *Physiol. Rev.* **54,** 418–540.
6. Wu, J. Y., Chude, O., Wein, J., Roberts, E., Saito, K. & Wong, E. (1978) *J. Neurochem.* **30,** 849–857.
7. Ribak, C. E., Vaughn, J. E., Saita, K., Barber, R. & Roberts, E. (1976) *Brain Res.* **116,** 287–298.
8. Oertel, W. H., Tappaz, M. L., Kopin, I. J., Ransom, D. H. & Schmechel, D. E. (1980) *Brain Res. Bull.* **5,** Suppl. 2, 713–719.
9. Tomimatsu, U., Jansen, E. F., Gaffield, W. & Olsen, A. C. (1971) *J. Colloid Interface Sci.* **36,** 51–57.
10. Avrameas, S., Ternynck, T. & Guesdon, J.-L. (1978) *Scand. J. Immunol.* **8,** Suppl. 7, 7–23.
11. Vaitukaitis, J.-L., Robbins, J. B., Nieschlong, E. & Ross, G. T. (1971) *J. Clin. Endocrinol.* **33,** 988–991.
12. Geffard, M., Puizillout, J.-J. & Delaage, M. A. (1982) *J. Neurochem.* **39,** 1271–1277.
13. Sternberger, L. A. (1979) *Immunocytochemistry* (Wiley, New York), 2nd Ed.
14. Buijs, R. M. (1982) in *Immunocytochemistry and Its Application in Brain Research*, eds. Van Leeuwen, F. W., Swaab, D. F. & Buijs, R. M. (Eur. Mol. Biol. Organ., Amsterdam), pp. 49–55.
15. Storm-Mathisen, J., Leknes, A. K., Bore, A. T., Vaaland, J. L., Edminson, P., Hang, F.-M. S. & Ottersen, O. P. (1983) *Nature (London)* **301,** 517–520.
16. Landsteiner, K. (1945) *The Specificity of Serological Reactions* (Harvard University Press, Cambridge, MA), 2nd Ed.